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Abstract:

Context: The endocrine function of human fetal adrenals (HFA) is activated already during first trimester, but changes in adrenal steroidogenesis during fetal life are not well characterized.

Objective: This study aimed to investigate HFA steroidogenesis by analyzing adrenal glands from 1st and 2nd trimester.

Design and Setting: Male and female HFA samples from gestational week (GW) 8-19 were examined, including a total of 101 samples from 83 fetuses.

Main Outcome Measure(s): Expression level of steroidogenic genes and protein expression/localization were determined by quantitative PCR and immunohistochemistry, respectively, and intra-adrenal steroid levels were quantified by LC-MS/MS.

Results: Transcriptional levels of *StAR, CYP11A1, CYP17A1, CYP21A2, CYP11B1/2* and *SULT2A1* was significantly higher in 2nd trimester compared with 1st trimester ($P<0.05$), while expression levels of *3β-HSD2* and *ARK1C3* were unaltered between GW 8-19. All investigated steroidogenic proteins were expressed in a distinct pattern throughout GW 8-19 with most enzymes expressed primarily in the fetal zone, except 3β-HSD1/2 which was mainly expressed in the definitive zone. The abundant steroidogenic enzyme expression was reflected in overall high intra-adrenal tissue concentrations of mineralocorticoids, glucocorticoids, and androgens; cortisol was the most abundant (1,071-2,723 ng/g tissue, in average) and testosterone levels the lowest (2-14 ng/g tissue, in average).

Conclusions: The expression profiles of HFA steroidogenic enzymes are distinct from 1st to 2nd trimester, with no major differences between male and female samples. The intra-adrenal steroid hormone concentrations confirms that cortisol is produced throughout 1st and 2nd trimester, suggesting continued regulation of the HPA axis during this entire period.

Introduction

The morphology of the human fetal adrenal (HFA) gland differs from the adult organ. Thus, the morphology of the HFA gland is established around gestational week (GW) 7 where the adrenal cortex has differentiated from

adreno-genital primordial cells into a distinct morphology consisting of two separate zones; a thin outer definitive zone (DZ) and an inner fetal zone (FZ) that accounts for 80-90% of the adrenal tissue (1). During 2nd trimester an additional transitional zone (TZ) develops at the interface between the DZ and FZ after which the morphology of the HFA gland is unchanged until birth (1).

Activation of the hypothalamus-pituitary-adrenal (HPA) hormone axis is essential for adrenal steroidogenic enzyme expression (2). Thus, activation of the adrenal melanocortin receptor 2 (MC2R) through binding of pituitary-secreted adrenocorticotropic hormone (ACTH) is necessary for the endocrine capacity of the HFA gland (1,3,4). The FZ (and later TZ) is described as the most steroidogenic active zone expressing Steroidogenic acute regulatory protein (StAR), Cytochrome P450 11A1 (CYP11A1), Cytochrome P450 17A1 (CYP17A1), Cytochrome P450 21A2 (CYP21A2), Cytochrome P450 11B (CYP11B1/ CYP11B2) and Sulfotransferase Family 2A Member 1 (SULT2A1) from around GW 7 (4,5,6). Throughout gestation CYP17A1 and SULT2A1 are exclusively localized to the FZ and TZ (1,7-9) while CYP11A1, CYP21A2 and StAR are also expressed in the DZ from around GW 23 (7,8,10,11).

3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) mediates the adrenal *de novo* synthesis of Δ^4 steroid hormones. Since the adrenal expression of *3β-HSD1* is very low compared to *3β-HSD2* (4,12) the detection of 3β-HSD1/2 in this study is thought mainly to represent expression of 3β-HSD2. In contrast to the other HFA steroidogenic enzymes, 3β-HSD2 is mainly expressed in DZ cells from GW 7-10 (3,4,7,8,11). However, a recent study showed a constant 3β-HSD2 expression throughout 2nd trimester (13). This finding suggests that the 3β-HSD2 enzyme in addition to its role in the regulation of fetal adrenal cortisol-mediated negative feedback on the HPA axis to minimize adrenal androgen synthesis during the critical time window of sex differentiation (4,14-16) also may act as a negative regulator throughout 2nd trimester.

HFA steroidogenesis is tightly regulated throughout the 1st and 2nd trimester which is crucial as the adrenal steroid hormones affect the overall endocrine intrauterine environment from early fetal development. Elevated levels of HFA androgens can be a consequence of dysregulated adrenal steroid pathways, in which imbalanced steroidogenesis can cause masculinization of the external genitals in female fetuses with

89 congenital adrenal hyperplasia (17). However, HFA steroidogenesis is not well characterized during fetal
90 development as previous studies investigating the steroidogenic function have focused on either 1st or 2nd/3rd
91 trimester only, characterizing selected adrenal steroidogenic enzymes. This study therefore aimed to collect
92 detailed expression-data of all the classical steroidogenic enzymes and determine the intra-adrenal steroid
93 levels from 1st and 2nd trimester HFA's in one inclusive study. Thus, this study is the first to examine both
94 human fetal-adrenal-tissue steroid levels, gene and protein expression of steroidogenic enzymes throughout
95 1st and 2nd trimester fetal development.

96 Material and Methods

97 *Collection of human fetal adrenals and ethical approvals*

98 Human fetal adrenal tissue from 1st trimester (8-12 GW) and 2nd trimester (14-19 GW) were isolated from
99 material available following elective surgical termination of pregnancy at Copenhagen University Hospital
100 (Rigshospitalet) and Hvidovre Hospital (Denmark) as well as the Royal Infirmary of Edinburgh (UK) and the
101 Human Developmental Biology Resource (UK). A total of 101 HFA samples were used in this study collected
102 from 83 individual fetuses as two adrenals from the same fetus was occasionally used in different analyses.
103 The study is approved by the Danish regional ethics committee (H-1-2012-007) and the UK Lothian Research
104 Ethics committee (LREC08/S1101/1). Woman gave their informed written and oral consent. None of the
105 terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Fetal age
106 was determined by scanning crown-rump length and confirmed by evaluation of foot length (18). Hence, fetal
107 age will be about 2 weeks greater than studies reporting days/weeks post conception. After dissection, adrenal
108 tissue samples were either snap frozen at -80 °C or fixed immediately in 4% buffered formalin. Fetal sex
109 determination was based on PCR using specific primers targeting *SRY.* DNA for sex determination was
110 extracted from fetal limb tissue and isolated with NucleoSpin Genomic DNA as described by the manufacture
111 (Macherey-Nagel, Düren, Germany). PCR cycle conditions: one cycle of 3 min at 95°C; 25 cycles of 30 sec at
112 95°C, 1 min at 60°C, 1.5 min at 65 °C, and one cycle of 5 min at 72 °C.

Gene expression

Total RNA was extracted from one frozen HFA glands in samples from GW 8-12 (11 male and 11 female, collected from 22 fetuses), while half of a frozen HFA gland was used in samples from GW 14-19 (9 male and 8 female, collected from 17 fetuses and isolated using the NucleoSpin RNA II purification kit, according to the manufactures instructions (Macherey-Nagel, Düren, Germany). cDNA was synthesized using a dT20 primer and random hexamers. Real time polymerase chain reaction (RT-PCR) was performed using specific primers targeting preselected mRNAs. All primers were designed to span intron-exon boundaries with optimal annealing temperatures ~62 °C, comparable primer length and CG contents (Table 1). All amplicons were initially verified by sequencing (Eurofins MWG GmbH, Germany) while primer amplification efficiency and detectable dynamic range of all primer-sets was validated prior to the analysis of the HFA samples. RT-PCR cycle conditions: one cycle of 3 min at 95°C; 40 cycles of 30 sec at 95°C, 1 min at 62°C, 1 min at 72°C, and one cycle of 5 min at 72°C. Quantitative RT-PCR analysis was performed in triplicates using Brilliant II SYBER Green qPCR Master mix (Agilent technologies). Changes in gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method (19). The expression levels were normalized to the reference gene, *RSP20,* and calculated as a ratio with male or female GW 8-9 set to 1 in respective samples.

Immunohistochemistry

Adrenal tissue from GW 8-12 (13 male and 13 female, collected from 26 fetuses) and GW 14-19 (10 male and 6 female, collected from 16 fetuses) were used for immunohistochemistry. Formalin fixed adrenal tissue were dehydrated, paraffin embedded and sectioned (4 µm) using standard procedures. Primary antibodies, dilutions and retrieval buffers are listed in Table 2. Immunohistochemistry (IHC) staining was initially conducted according to a standard protocol as described previously (20). Subsequently, the protocol was modified to include antigen retrieval by pressure cooker as previous described (21). In brief, tissue sections were subjected to heat-induced antigen retrieval buffer in a pressure cooker and endogenous peroxidase was blocked with 3% (v/v) H_2O_2 in MeOH for 30 min. Between each step sections were washed in Tris-buffered Saline (TBS). Sections were incubated in 5% bovine serum albumin (BSA w/v) in Horse serum (20% v/v ImmPRESS) and TBS (80% v/v) or 0.5% milk powder for 30 min depending on optimization for each antibody.

Sections were subsequently incubated overnight with primary antibody diluted in serum at 4°C in a humidified chamber followed by 1h at room temperature. Sections were then incubated for 30 min with the appropriate ImmPRESS HRP (peroxidase) secondary antibody diluted in normal serum. Visualization was performed using ImmPACT AEC peroxidase substrate (Vector Laboratories, Burlingame, CA, USA). Included negative controls replaced the primary antibody with dilution buffer only, none of which showed any staining. All sections were counterstained with Meyer's hematoxylin before mounting with Aquatex (Merck, Damstad, Germany).

Quantification of stained cells

Two independent investigators evaluated all stainings. Sections were first investigated manually on a Nikon Microphot-FXA microscope, subsequently slides were scanned on a Nano-Zoomer 2.0 HT (Hamamatsu Photonics, Herrsching am Ammersee, Germany) and analyzed using the software NDPview version 1.2.36 (Hamamatsu Photonics). The intensity of immunoreactivity was classified according to a pre-defined scoring system: ++, strong staining in all cells of a given type in the sample; ++/+, strong staining prevalent, but some weakly stained cells also visible; ++/-, strong staining present, but negative cells also present; +/++, majority of cells weakly stained, but some strong staining present; +/++/-, heterogeneous pattern with a mixture of strongly positive, weakly stained, and negative cells; +, (1) weak staining overall or (2) strong staining in a small number of cells; +/-, weak staining in limited areas; -/+, weak staining in single cells.

Quantification of steroid hormones

Tissue samples from intact HFA glands GW 8-12 (10 male and 10 female, collected from 20 fetuses) and halved HFA glands GW 14-19 (9 male and 8 female, collected from 17 fetuses) were included for the intra-adrenal steroid analysis by LC-MS/MS measurements. Tissue samples were weighed (1.7 mg - 43 mg wet weight) and ground in 1 mL 80% (v/v) MeOH. The homogenate was transferred to glass tubes evaporated to almost dryness under a N_2 stream. Internal standard stock solution (5-500 ng/mL in 100 µL) was added to each sample pellet as previously described (22) and subsequently 375 µL 1M ammonium acetate buffer (pH 5.5) was added. Next, 2 mL Heptan: Ethylacetat 3:2 (v/v) was added and samples were transferred to eppendorf tubes. Samples were then shaken for 15 min followed by 10 min of centrifugation at 2000 x g (4°C). Each tube was transferred to a dry-ice bath (dry-ice pills in ethanol (99%) for a few minutes to freeze the

aqueous phase followed by decantation of the organic phase to a new glass tube. The organic phase was evaporated to dryness under a stream of N_2 and finally, the steroids were resolved in an appropriate amount of 50% (v/v) MeOH (tissue GW 8-12: 100 µL, tissue GW 14-19; 200 µL) for LC-MS/MS analysis as previously described (22). All samples were measured in one single batch which includes standards for calibration curves, unknown samples, two blanks and for method control; three un-spiked human serum pool samples and three serum pool samples spiked with low and high levels, respectively.

Statistical analysis

qPCR and LC-MS/MS data were statistically analyzed for age and sex specific differences. Age differences were tested by the nonparametric Mann-Whitney test in which HFA age groups GW: 10-12, GW: 14-16 and GW:17-19 were compared with male GW 8-9. Sex differences were also tested by nonparametric Mann-Whitney test within each age group. $P<0.05$ was considered statistically significant.

Results

Gene expression patterns of adrenal steroidogenic enzymes

The selected steroidogenic enzymes were expressed in all investigated HFA glands at the transcriptional level. Gene expression patterns were investigated separately in male and female samples, which were divided into four age groups: GW 8-9, GW 10-12, GW 14-16, and GW 17-19. Expression levels were calculated as a ratio of levels relative to male GW 8-9 (reference value set to 1). No sex differences were observed in the transcription levels of the examined steroidogenic enzymes nor in the transcription levels of the ACTH receptor, MC2R, throughout the investigated developmental period (Figure 1). During 2nd trimester, the expression level of *StAR, CYP11A1, CYP17A1, SULT2A1* and *MC2R* increased approximately 10-fold, while the expression level of *CYP21A2* and *CYP11B1/2* increased even further by >50-fold and >20 fold, respectively (Figure 1e-f). Only two of the investigated steroidogenic enzymes were constitutively expressed throughout 1st and 2nd trimester, namely: *3β-HSD2* and aldo-keto reductase family I member C3 (*ARK1C3,* also known as 17βHSD3) (Figure 1d,g). Additionally, the absolute gene expression levels of *3β-HSD2* and *ARK1C3* were lower compared to the rest of the steroidogenic enzymes (Figure 1j).

Distribution of steroidogenic enzymes in the human fetal adrenal cortex

The morphology of fixed adrenal samples included in the study was thoroughly examined. All samples appeared morphologically normal containing distinct zones in accordance with the gestational age of the developing HFA gland (Figure 2). Sections were stained with hematoxylin and eosin as well as Meyer's hematoxylin. The DZ consisted of small cells, while FZ cells were larger with a high content of cytoplasm (Figure 2c). As expected, the TZ was detected in 2nd trimester samples and consisted of a mixture of small distinct DZ cells and larger distinct FZ cells (Figure 2d) as previous described (1).

The expression profiles of steroidogenic proteins were analyzed using IHC on fixed adrenal samples. No differences between male and female samples were observed regarding the level of expression and the cell-type specific enzyme localization. Therefore, only male samples are shown for the age groups GW 8-9, GW 10-12, GW 14-16, and GW 17-19 in Figure 3. Adrenal zone-specific protein expression, localization, and the level of expression were evaluated based on a predefined scale and are summarized in Table 3.

Most of the investigated steroidogenic enzymes were abundantly expressed in the FZ throughout development of both 1st and 2nd trimester (Figure 3). More specifically, CYP11A1, CYP21A2 and SULT2A1 were abundantly expressed in the cytoplasm of 1st trimester FZ cells and weakly expressed in a limited number of DZ cells towards late 1st and throughout 2nd trimester. CYP17A1 showed a similar expression and localization pattern, although CYP17A1 was expressed specific in the FZ except for a few strongly-positive cells scattered within the DZ in both 1st and 2nd trimester samples. During 2nd trimester, protein expression of CYP17A1 increased around the TZ as it decreased in the cells of the inner FZ. In contrast, 3β-HSD1/2 was the only observed steroidogenic enzyme which was frequently expressed in a limited number of DZ cells and only very few FZ cells. During 2nd trimester 3β-HSD1/2 was still abundantly expressed though only in a sub-population of DZ and FZ cells compared with 1st trimester samples. Like the steroidogenic enzymes, the ACTH receptor MC2R was also detected throughout the investigated period of fetal development. More specifically, weak cytoplasmic MC2R expression was detected throughout 1st and 2nd trimester, but nuclear MC2R expression was also occasionally found in late 1st trimester and throughout 2nd trimester. Therefore, the MC2R expression

patterns observed in this study should be interpreted with caution due to the unexpected nuclear expression (23), which is most likely an artifact of the antibody.

Human fetal adrenal Steroid tissue concentrations

The endocrine activity of the HFA was further evaluated by determination of the intra-adrenal steroid concentrations. Thus, steroids were extracted from adrenal gland tissue followed by LC-MS/MS determination of mineralocorticoid, glucocorticoid and androgen levels. Samples were divided according to sex and age (as described previously). All investigated mineralocorticoids, glucocorticoids and androgen metabolites were detected in HFA tissue from GW 8-19.

The measured concentrations of mineralocorticoid metabolites progesterone and corticosterone were in general equivalent with no significant difference in relation to neither gestational age nor sex. The only exception was a significant age-related increase in female corticosterone levels at GW 17-19 compared with male tissue concentrations at GW 8-9 (Figure 4a). The intra-adrenal levels of glucocorticoids 17OH-progesterone was unaltered over the investigated developmental period with no differences between male and female adrenal tissue samples were detected. In contrast, both 11-deoxycortisol, cortisol and cortisone, a significant increase in intra-adrenal steroid concentrations was found at GW 17-19 compared with male GW 8-9 (Figure 4b). For 11-deoxycortisol a significant increase in intra-adrenal steroid concentrations was further observed at GW 10-12 and GW 14-16, the latest only in male samples. The only observed sex-specific difference was found in the intra-adrenal cortisone concentrations which were significantly higher in female samples at GW 8-9 compared with age matched males (Figure 4b). Interestingly, cortisol levels were the highest of all measured intra-adrenal steroid hormones throughout the investigated developmental period. Hence, cortisol tissue concentrations were approximately 2-fold higher than those of 17OH-progesterone and 11-deoxycortisol and approximately 10-fold higher than cortisone (Figure 4b).

Similar to the trend for the majority of measured steroid hormones, the adrenal tissue concentration of androgens was relatively constant throughout GW 8-19, only in male GW 14-16 samples a significant increase in testosterone levels were found when compared with male GW 8-9 tissue concentrations (Figure 4c). The

adrenal conversion of androstenedione to testosterone appeared to be limited as tissue concentrations of androstenedione were approximately 80-fold higher than testosterone (Figure 4c).

Discussion

This study investigated the adrenal steroidogenic expression pattern during the 1st and 2nd trimester of human fetal development. HFA glands function as active endocrine organs from early fetal development, which was confirmed by this study demonstrating expression of all investigated steroidogenic enzymes both at the gene and protein level in all investigated samples from GW 8-19. The distinct morphological expression pattern of the investigated enzymes is in accordance with previous studies focusing on either 1st or 2nd trimester human adrenals (4,7,8,10,11,13,14). Thus, our data support the prominent hypothesis of the FZ, and later in development also the TZ, being the main site of *de novo* steroid synthesis in the HFA. Interestingly, the transcription of steroidogenic genes appeared to be regulated differently with unaltered expression of *ARK1C3* and *3β-HSD2* during the investigated period while the expression of the other steroidogenic enzymes (*StAR, CYP11A1, CYP17A1, CYP21A2, CYP11B1/2,* and *SULT2A1)* and the ACTH receptor *MC2R* were significantly increased in 2nd trimester. The observed increases in gene expression levels in 2nd trimester was not evident at the protein level; presumably due to the lack of sensitivity of immunohistochemistry to detect differences in expression level. However, the genes upregulated during 2nd trimester were also the steroidogenic enzymes that showed the most abundant immunostaining in the FZ while the low constitutively expressed 3β-HSD1/2 was only detected in a small subpopulation of cells mainly located in the DZ. Furthermore, the up-regulation in gene expression for the majority of the investigated steroidogenic enzymes was not reflected in higher tissue concentrations of steroidogenic hormones which overall did not differ between 1st and 2nd trimester (Figure 5b). It remains to be examined whether the 2nd trimester transcriptional upregulation of steroidogenic enzymes are reflected in steroid levels secreted by the HFA to the fetal circulation, which were not possible to determine in this study.

The constitutive expression of 3β-HSD1/2 throughout 1st and 2nd trimester of human fetal development in male and female HFA samples is in contrast with the previous reported transient expression profile (3,4,7,8,11). Thus, our results demonstrate that 3β-HSD1/2 is highly expressed in a sub-population of DZ cells from GW 8-19, which is in accordance with recent published data (13). Even though the percentage of 3β-HSD1/2 positive cells in DZ and FZ appeared to decrease during 2nd trimester, this study is the first to report a small number of 3β-HSD1/2 positive FZ cells in 2nd trimester fetuses up to GW 19. In general, the small number of 3β-HSD1/2 positive cells compared with the abundant expression of the majority of the adrenal steroidogenic enzymes is consistent with the reported low *3β-HSD2* transcripts levels in this study as well as previous reports (3,4,11,13). The immediate imbalance between the low 3β-HSD1/2 expression and high tissue concentrations of the 3β-HSD2 catalyzed Δ^4 steroids: progesterone, 17OH-progesterone, and androstenedione, has previously been suggested to be the result of either circulating placental progesterone or the transfer of intermediates of the steroid pathways between the adrenal zones (13). Since it has been shown by double immunofluorescence staining in 2nd trimester HFAs, CYP11A1 and CYP21A2 only co-localize in a limited number of FZ cells (13) this could support the hypothesis of transport of steroidogenic intermediates between the adrenal zones. Moreover, the presence of high levels of intra adrenal androstenedione in 2nd trimester determined in this study favors 3β-HSD2 dependent *de novo* synthesis, as 17OH-progesterone is a poor substrate for human CYP17A1 (C17,20 lyase) (24) indicating that adrenal androstenedione is not likely generated from placental progesterone.

The HFA's produce androgens throughout 1st and 2nd trimester in both male and female samples. The detection of high HFA tissue concentrations of androstenedione and low levels of testosterone indicate that this step-in steroidogenesis is tightly regulated. The low intra-adrenal testosterone levels detected from GW 8-19 are in accordance with previous studies that have examined both fetal adrenal tissue concentrations and secreted levels in organ culture (3,4,13). Furthermore, the low tissue concentrations of testosterone are also in accordance with the relatively low transcriptional expression level of *ARK1C3* which mediates adrenal *de novo* testosterone synthesis. The expression level of *ARK1C3* in 1st trimester samples detected in this study supports previous investigations (3,4) while *ARK1C3* expression levels, to our knowledge, have not been investigated previously in 2nd trimester HFA tissue. Consequently, the significant increase in male testosterone

297 levels at GW 14-16, compared with male GW 8-9, is inconsistent with the observed overall unaltered
298 expression level of *ARK1C3* suggesting that testosterone levels are not only regulated via gene transcription.
299 The observed peak in male testosterone around GW 14-16 is not significantly different from the female GW
300 14-16 age group and it is likely that the adrenal testosterone production is considerably lower than the
301 contribution from fetal testis (25). Moreover, this study is the first to report androstenedione as the most
302 abundant Δ^4 adrenal androgen synthesized by the classical steroidogenic pathway throughout 1st and 2nd
303 trimester in HFA, which is in contrast to previous observations of higher adrenal androgen production during
304 the 1st trimester (13).
305
306 The HFA produces cortisol throughout 1st and 2nd trimester indicating continued regulation of the HPA axis
307 during this entire period. In line with this finding is the detection of cytoplasmic MC2R expression from GW 8,
308 which is in accordance to previous studies (3,4). However, due to the unexpected nuclear expression pattern
309 of MC2R observed in this study these results should be interpreted with caution as MC2R is known to be
310 expressed in the cytoplasm (at the endoplasmic reticulum) until its translocated with MRAP to the plasma
311 membrane to mediate ACTH signaling (23).The abundant HFA tissue concentrations of cortisol detected in
312 this study further supports the recent hypothesis that cortisol-mediated negative feedback of the HPA axis is
313 not restricted to the 1st trimester, but extends through the 2nd trimester (13) including GW 14-19. Detection of
314 intra-adrenal cortisol in 2nd trimester is consistent with the observed expression of 3β-HSD1/2 and in
315 accordance with a previous study detecting cortisol in HFA tissue from 2nd trimester (13) although the cortisol
316 concentrations measured in the present study are approximately 10-fold higher than the concentrations
317 previously reported. During GW 8-14 cortisol mediated negative feedback on the HPA axis is thought to be
318 crucial since this period is the window of development in which the external genitalia differentiates (15,16).
319 Hence, dysregulated adrenal steroidogenesis resulting in imbalance with excess of adrenal androgens and
320 reduced cortisol in this time window can thus cause virilization of female genitalia in fetuses with CAH (17).
321 Since adrenal androgens can be converted to estrogens by the placental aromatase (CYP19A1) later in
322 pregnancy, the adrenal secretion of androgens during 2nd trimester is thought to have less effect on genital
323 differentiation (15,16). However, the observed abundant concentration of cortisol throughout 1st and 2nd
324 trimester raises new questions about whether alterations in cortisol production could still have an impact on

virilization of CAH fetuses during 2nd trimester or may possibly influence other events during human fetal development.

This study provides a detailed characterization of both male and female adrenal endocrine function during 1st and 2nd trimester of human fetal development. It is evident from the gene and protein expression pattern of steroidogenic enzymes as well as the steroid measurements in tissue that the HFA functions as a steroidogenic active organ from early development by producing high levels of mineralocorticoids, glucocorticoids and androgens. Even from GW 8 we report a distinct expression pattern for the investigated adrenal steroidogenic enzymes with a significant increase of gene expression in 2nd trimester samples for the majority of enzymes, with exception of the unaltered expression of *3β-HSD2* and *ARK1C3*. Based on the intra-adrenal steroid hormone concentrations we found that the androstenedione is the most abundant Δ^4 adrenal androgen synthesized via the classical steroidogenic pathway throughout 1st and 2nd trimester. Additionally, this study confirms that cortisol is produced throughout 1st and 2nd trimester, suggesting continued regulation of the HPA axis during this entire period.

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Author contributions

C.M., A.Ju., A.Jø. conceived and designed the experiments. C.M., J.E.N., H.F. performed the experiments. K.K., S.P., L.L., L.L.T., K.J.M. provided study material. C.M., J.E.N., H.F., A.M.A., R.T.M., A.Ju., A.Jø. analyzed data. C.M., A.Jø. wrote the manuscript. All authors read and approved the submitted version of the manuscript.

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Abbreviations: ARK1C3: aldo-keto reductase family I member C3; 3β-HSD2: 3β-hydroxysteroid

dehydrogenase type 2; CHA: congenital adrenal hyperplasia; CYP11A1: cytochrome P450 11A1; CYP11B1/2: cytochrome P450 11B; CYP17A1: cytochrome P450 17A1; CYP21A2: cytochrome P450 21A2; DHEAS: dehydroepiandrosterone sulfate; DZ: definitive zone; FZ: fetal zone; GW gestational week; HFA: human fetal adrenal; HPA: hypothalamus-pituitary-adrenal; IHC: immunohistochemistry; MC2R: melanocortin receptor; StAR: steroidogenic acute regulatory protein; TZ: translational zone.

Figure 1. Gene Expression Level of Human Fetal Adrenal Steroidogenic Enzymes during 1st and 2nd Trimester

(a-i) qRT-PCR analysis of a range of steroidogenic associated enzymes and receptors in male and female human fetal adrenal samples divided into four age groups: GW 8-9, GW 10-12, GW 14-16, and GW 17-19. Expression is relative to the reference gene *RSP20*. Expression level is set to 1 in male GW 8-9 samples. In total 39 adrenal samples were used. Bars represent mean +/- SEM with individual data points shown as blue triangles and red circles, male and female samples respectively, *n*=3-6. Differences in age compared with male GW8-9 indicated as significantly different * (P<0.05), **(P<0.01). (j) Overall human fetal adrenal transcript levels from 1st and 2nd trimester samples, n=39. Data points represent –ΔCt values (relative to housekeeping gene *RSP20*) of investigated gene transcript levels in individual adrenal samples (male and female). Error bars represent mean ± SD.

Figure 2. Human Fetal Adrenal Gland Morphology and Zonation

Schematic illustration of the morphological distinct zonation in (a) 1st trimester human fetal adrenals (GW 7-12) and (b) 2nd trimester human fetal adrenals (GW 14-19) viewed as a cross section of the gland. (c) HE and Mayer stainings of a 1st trimester human fetal adrenal gland. (d) HE and Mayer stainings of a 2nd trimester human fetal adrenal gland, scale bar corresponds to 100 µm. DZ: definitive zone TZ: transitional zone, FZ: fetal zone.

Figure 3. Expression of Steroidogenic Enzymes in 1st and 2nd Trimester Human Male Adrenal Glands

Immunohistochemical staining of serial sections for: CYP11A1, CTP17A1, 3β-HSD1/2, CYP21A2, CYP11B1 and SULT2A1 (steroidogenic proteins) as well as MC2R (ACTH receptor) in male samples. No differences were observed between male and female samples within a given gestational group and therefore only male samples are shown. Scale bars = 100 µm.

Figure 4. Tissue Levels of Steroid in 1st and 2nd Trimester Human Adrenals

LC-MS/MS measurements (ng/g wet tissue) of secreted adrenal steroid hormones determined from male and female adrenal tissue extracts. Bars represent mean +/- SEM with individual data points shown as blue triangles and red circles, male and female samples respectively, *n*=3-5. Differences in age compared with male GW8-9 indicated as significantly different * ($P<0.05$). Differences between sex within the same age group are indicated as significantly different ¤ ($P<0.05$).

Figure 5. Overview of Human Fetal Adrenal Steroidogenic Development

(a) Schematic overview of adrenal steroidogenesis. Gene transcripts of enzymes significantly upregulated in 2nd trimester is shown in green boxes, with unaltered gene transcripts shown in red boxes. Measured steroid hormones are shown in the bold text font. (b) Overview of steroid hormone levels relative to progesterone levels throughout the investigated developmental period. Data are shown as mean values fetal adrenal samples (for SEM values see data in Figure 4).

Table 1. **Primers used for RT-PCR and Quantitative PCR**

Gene	***HGNC ID***	**Forward Primer**	**Reverse Primer**	**Application size**
StAR	11359	*CACCCCTAGCACGTGGATTA*	*CTTGGTTGCTAAGGATGCCC*	152 bp
CYP11A1	2590	*ATAAACCGACTCCACGTTGC*	*ACAATGGCTGGCTAAACCTG*	134 bp
CYP17A1	2593	*GAGTTTGCTGTGGACAAGGG*	*CGCTGGATTCAAGAAACGCT*	117 bp
3β-HSD2	5218	*CAGGCTCTTTTCAGGAATGG*	*CTTGGACAAGGCCTTCAGAC*	117 bp
CYP21A2	2600	*GAGTTCTGTGAGCGCATGAG*	*GAATCACGTCCACAATTTGGAT*	205 bp
CYP11B1/2	2591/2	*CTTCCACTACACCATAGAAGCCAGC*	*CCTCAAAGTGCTCCTTCCACAC*	200 bp
ARK1C3	386	*GGAGGCCATGGAGAAGTGTAAGGA*	*CCAGAGCACTATAGGCAACCAGAAC*	215 bp
SULT2A1	11458	*ACAGGACACAGGAAGAACCATAGAG*	*CTTCAGCTTGGGCCACTGTGAA*	230 bp
MC2R	6930	*ACATGGGCTATCTCAAGCCAC*	*TCCAGATGACCGTAAGCACCA*	204 bp
RSP20	10405	*AACAAGCCGCAACGTAAAATC*	*ACGATCCCACGTCTTAGAACC*	166 bp
SRY	11311	*GAATATTCCCGCTCTCCGGA*	*GCTGGTGCTCCATTCTTGAG*	470 bp

All primers are shown in 5’ to 3’ direction.

Table 2. Antibodies, Dilutions and Retrieval Buffers used

Antibody	Dilution	Retrieval buffer	Species	Supplier	Number
CYP11A1	1:10.000	TEG	Rabbit	Sigma	HPA016436
CYP17A1	1:1500	CIT	Rabbit	Abcam	Ab134910
3β-HSD1/2	1:6000	TEG	Rabbit	Gift from Ian Mason	-
CYP21A2	1:6000	TEG	Goat	Santa Cruz Biotechnology	Sc-48466
CYP11B1	1:3000	TEG	Mouse	Santa Cruz Biotechnology	Sc-374096
SULT2A1	1:6000	CIT	Rabbit	Sigma	HPA041487
MC2R	1:800	TEG	Rabbit	Santa Cruz Biotechnology	Sc-13107

Antigen retrieval was conducted by pressure cooking of the sections in indicated retrieval buffer for 30 min. in a decloaking chamber. TEG buffer: 10 mM Tris, 0.5 mM EGTA, pH 9.0; Citrate (CIT) buffer: 10 mM, pH 6.0.

Table 3. Analysis of Adrenal Zone-Specific Protein Localization and Level of Expression

	DZ				FZ				C
GW: / Enzyme:	8-9	10-12	14-16	17-19	8-9	10-12	14-16	17-19	17-19
CYP11A1	+(2)	+(2)	+(2)	+/++/-	++	++/+	++/+	++/+	
CYP17A1	-/+	+(2)	+(2)	+(2)	++/+	++/+	++ (TZ), ++/+(FZ)	++(TZ), ++/+(FZ)	
SULT2A1	+(2)	+(2)	+(2)	+(2)	++/+	++/+	++/+	++/+	
MC2R	+(1)	+/++	+/++, +/++/-(n)	+(1), ++/-(n)	+/++	+/++	+/++, -/+(n)	+/++, +/++/-(n)	++/-(n)
CYP11B1	+/-	-/+	-/+	-/+	+/-	+/-	+(1)	+(1)	
CYP21A2	-/+	+(2)	+(2)	+(2)	+/++	++/+	++/+	++/+	
3βHSD1/2	++/-	++/-	+/++/-	+/++/-	+(2)	+(2)	+(2)	+(2)	

GW: gestational week, DZ: Distal Zone, FZ: Fetal Zone, TZ: transitional zone, C: Capsule, n: nuclear staining.

n = 6-15 adrenals within each gestational group.

TZ staining is indicated when the TZ protein expression differed from the FZ expression in the 2nd trimester tissue (GW: 14-19).

Scale: ++, strong staining in all cells of a given type in the sample; ++/+, strong staining prevalent, but some weakly stained cells also visible; ++/-, strong staining present, but negative cells also present; +/++, majority of cells weakly stained, but some strong staining present; +/++/- , heterogeneous pattern with a mixture of strongly positive, weakly stained, and negative cells; +, (1) weak staining overall or (2) strong staining in a small number of cells; +/-, weak staining in limited areas; -/+, weak staining in single cells.

Figure 1

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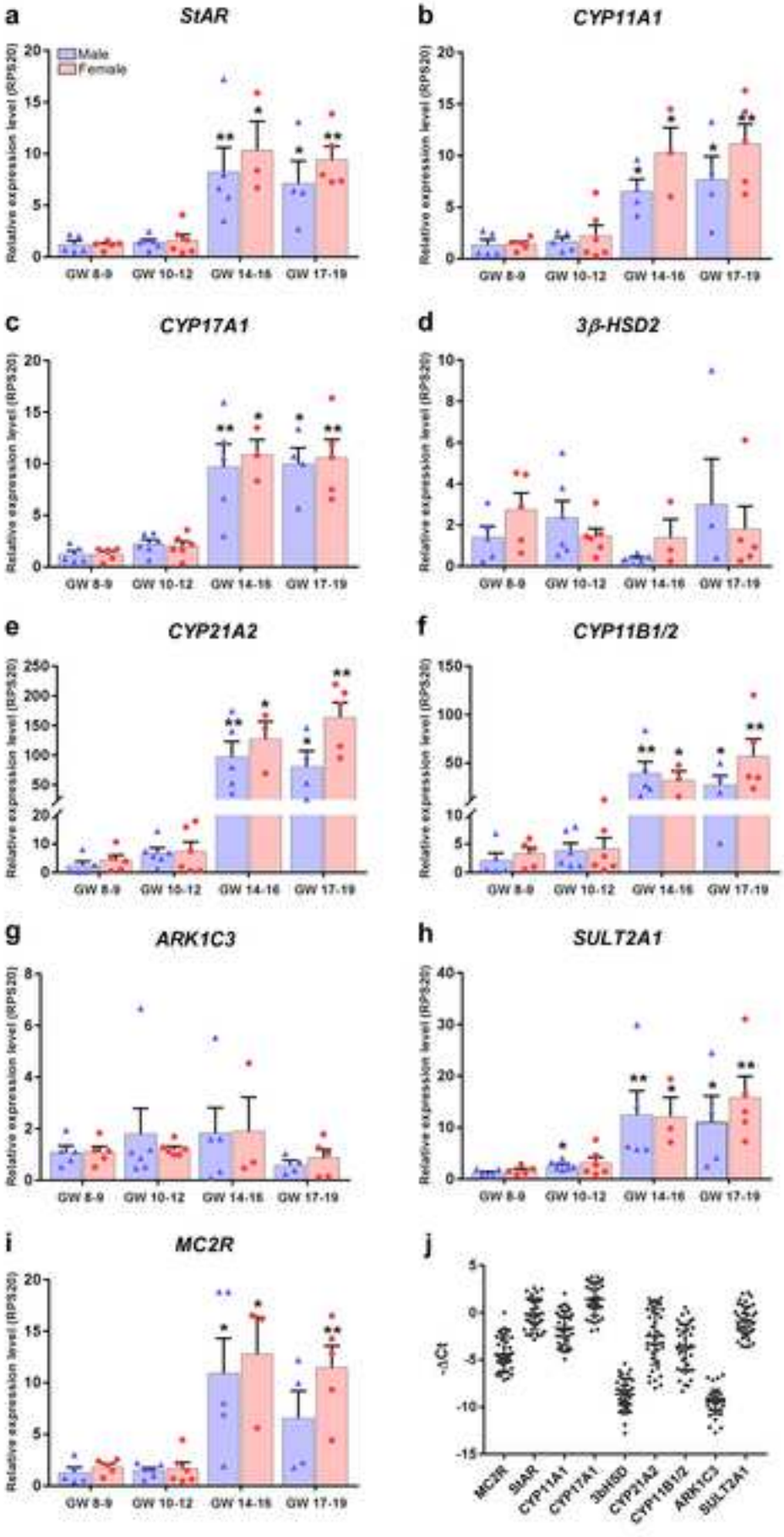

Figure 2

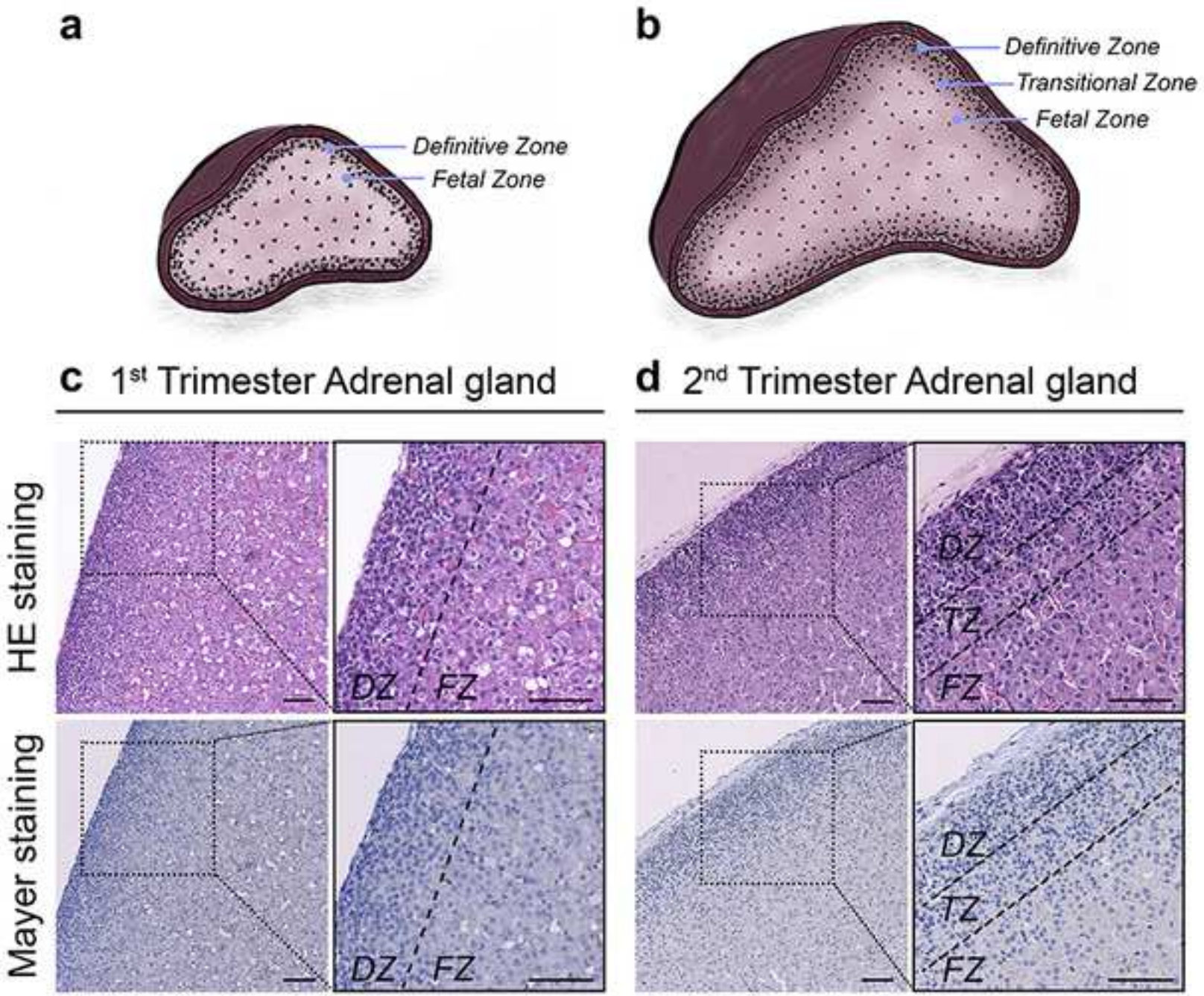

Figure 3

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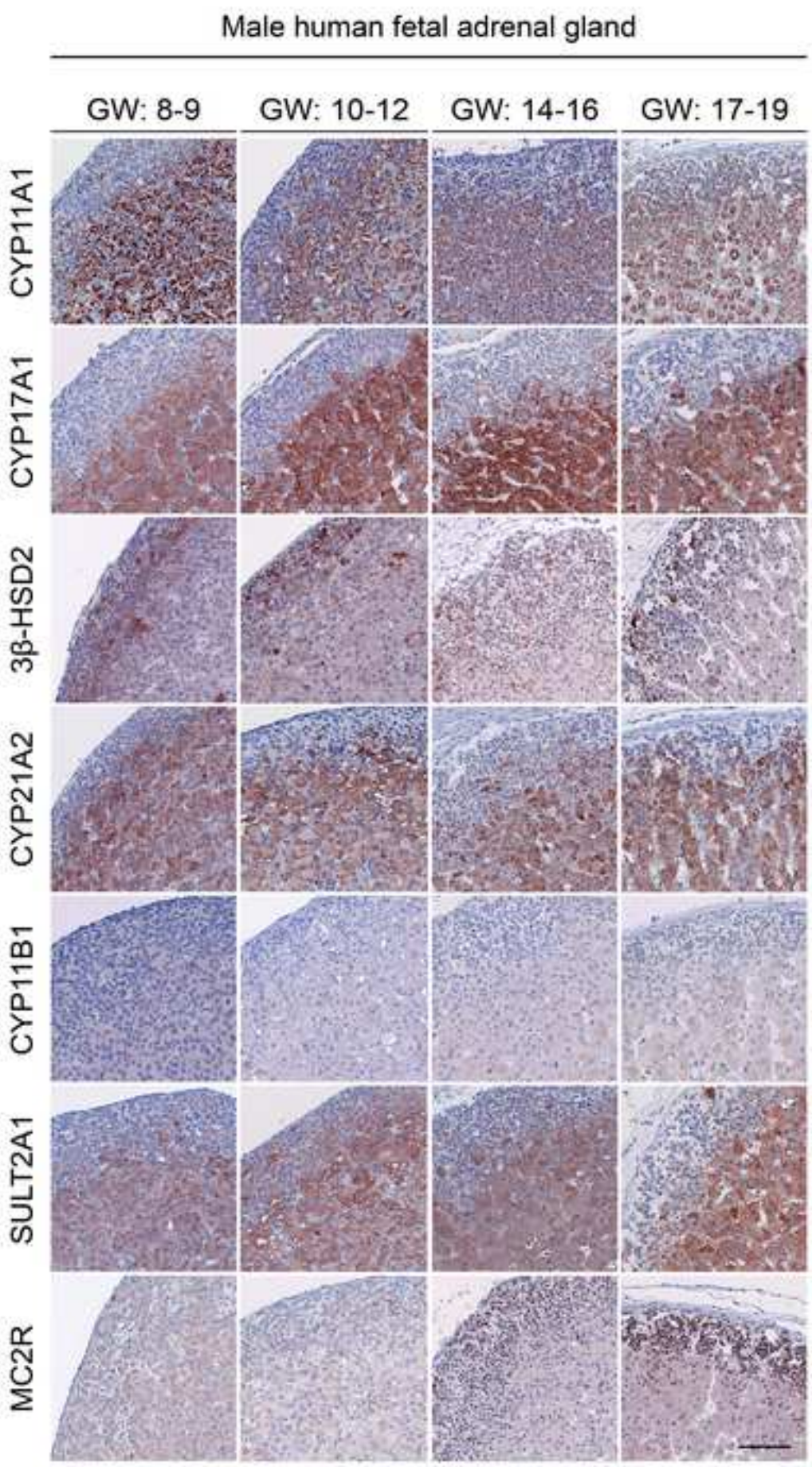

Figure 4

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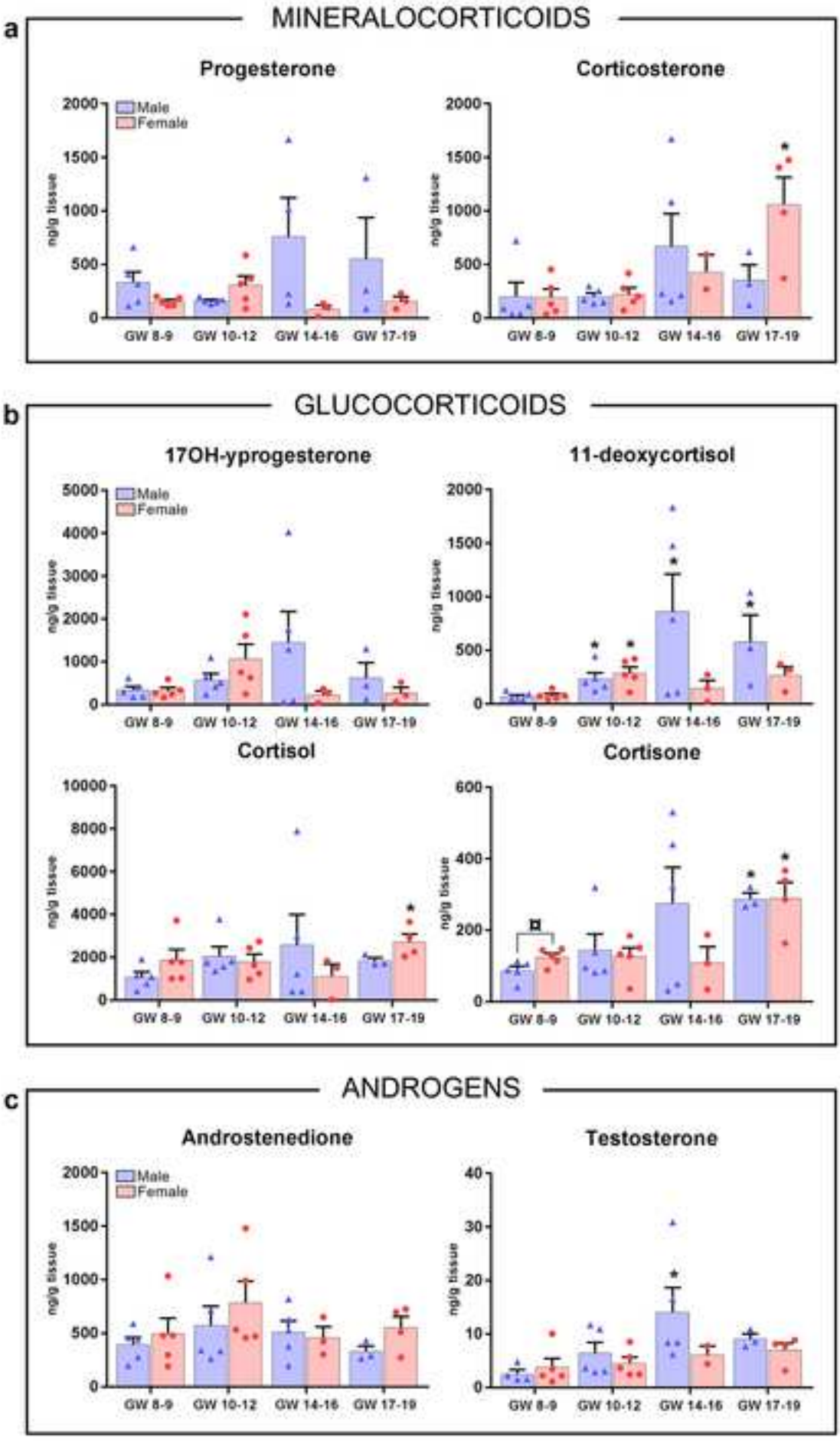

Figure 5

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